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BIOLOGICAL BEHAVIOUR OF GENITAL HPV INFECTIONS

A Prospective Follow-up and Partner Study.

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ABSTRACT

To assess the natural history of Human papillomavirus (HPV) infections in the lower female genital tract and their associations with intraepithelial neoplasia (CIN, VAIN, VIN) and genital squamous cell cancer, a long-term prospective follow-up study was started in October 1981. The project was the first of its kind, and still continues to be the largest (and oldest) ongoing prospective follow-up study in this field. The final number of women in the two Follow-up **Groups A and B**, is currently 530, including HPV-lesions with and without concomitant CIN (HPV-CIN, and HPV-NCIN lesions, respectively). The mean follow-up time of the whole series approaches 80 months by now. The recruitment of women into the third series, called the **Treatment Group**, currently comprising the same number (530) of patients as in Groups A & B, was discontinued in September, 1990, when the final size of the group was reached. During the current project period (**January-December 1992**), the recruitment of the male sexual partners of the Treatment Group women into the **Partner Group** has been continued. The number of the male partners enrolled has recently exceeded 300. **During the ongoing research period, the enrollment of a fifth series of patients was initiated, when small children and newborn babies born to mothers in the Follow-up and Treatment Groups are being recruited for clinical examination and sampling of their oral cavity and nasopharynx. The aim of this approach is to elucidate the possible non-sexual (i.e., vertical) transmission of HPV from the mother to her child and the biological significance of such an eventual transmission.** The follow-up of the women included in the two Follow-up Groups (A & B) as well as in the Treatment Group has been continued as done for over 10 years by now, using colposcopy, PAP smears and/or punch biopsy. The latter are examined by the techniques outlined in the Research Plan. These include histological assessment and grading as well as HPV typing by in situ hybridization and PCR. Cervical swabs were analysed for isolation of Chlamydia trachomatis and HSV, and serum samples for viral (HSV, CMV) serology. During the ongoing period, plans were made to initiate collaboration with German Cancer Reserch Centre to accomplish the serological analyses of the serum samples (thousands collected in the freezer between 1981-1992) for HPV type-specific antibody levels. The women in the Treatment Group are randomized according to four different treatment modalities; conization, laser therapy, cryotherapy, and interferon therapy. The HPV lesions of the male genitalia are examined by peniscopy, cytology, and biopsy (including HPV DNA typing and gene amplification by PCR), and treated by laser, whenever considered appropriate. The systematic HPV typing of the biopsies was continued using the in situ DNA hybridization (ISH) technique as a routine procedure. At the meantime, developmental work has been continued with the variety of DNA techniques to refine these methods applicable in routine laboratory praxis. As a result of this approach, a series of six commercial tests has been introduced into the market, which enable the HPV typing in both the biopsies and cell smears. During the current project period, biopsies have been tested for amplification of the cellular oncogenes (c-onc) by the ISH and PCR techniques. The role of p53 (a known antioncogene) has been assayed using immuno-histochemical staining in CIN lesions. As a new line of research, a series of samples has been analysed for mutations in p53 sequence by utilizing an automatic DNA sequencer. Binding of HPV E6 oncogen with p53 might have important implications in the malignant transformation by this virus. The complete HLA-typing of 100 follow-up women has been finally completed, and the results were submitted for publication. In the statistical treatment of the vast amount of data accumulated during these 10 years, a definite progress was made with the development of statistical models used to predict the disease outcome more accurately than possible at the moment. As a major part of the statistical analysis, the data derived from a case-control design (i.e., follow-up women and age-matched controls) were analysed to assess the factors increasing the risk for genital HPV infections. According to this analysis, the number of sexual partners during the past two years proved to be the single most important risk factor (RR >44 for women with 5 or more partners as compared to women with 1 partner only). The refinement of these data resulted in publication of yet another (MD) PhD Thesis (Dr. V. Kataja) in December. During the latter half of the period, a new technique was studied by Stina Syrjänen in DKFZ, Heidelberg (Prof. zur hausen); the organotypic culture (raft) of keratinocytes. This in vitro model will be established in our laboratory to be used in a number of different experiments, including the treatment trials with antisense oligonucleotides for HPV 16 E6/E7 mRNA.

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1.BRIEF SUMMARY

To assess the natural history of Human papillomavirus (HPV) infections in the lower female genital tract and their associations with intraepithelial neoplasia (CIN, VAIN, VIN) and genital squamous cell cancer, a long-term prospective follow-up was started in October 1981. The project was the first of its kind, and still is the most extensive (and of longest duration) ongoing prospective follow-up study in this field. The final number of women in the two Follow-up **Groups A and B**, is currently 530, including HPV-lesions with and without concomitant CIN (HPV-CIN, and HPV-NCIN lesions, respectively). The mean follow-up time of the whole series has exceeded 75 months by now. The recruitment of women into the third series, called the **Treatment Group**, currently comprising the same number (530) of patients as in Groups A & B, has been continued until September, 1990, when the final size of the group was reached. During the entire project period, the recruitment of the male sexual partners (of the Treatment Group women) into the **Partner Group** has been continued, their number currently reaching 265. The follow-up of the women included in the two Follow-up Groups A & B and in the Treatment Group was continued as done for 10 years by now, using colposcopy, PAP smears and/or punch biopsy. The latter are examined by the techniques outlined in the Research Plan (i.e., by light microscopy for HPV-induced changes and concomitant CIN, by immunohistochemistry for expression of HPV structural proteins, and immunocompetent cells using monoclonal antibodies to define B cells, T cell subsets, NK, natural killer cells and Langerhans cells). Cervical swabs were analysed for isolation of Chlamydia trachomatis and HSV, and serum samples for viral (HSV, CMV) serology. Blood samples have been also collected for analysis of T-cell subsets by flow cytometry (FACS). The patients in the Treatment Group were randomized according to four different treatment modalities; conization, laser therapy, cryotherapy, and interferon therapy with a cream. The HPV lesions of the male genitalia were examined by colposcopy, cytology, and biopsy (including HPV DNA typing), and treated by laser, whenever appropriate. The systematic HPV typing of the biopsies was continued using the in situ DNA hybridization technigue as a routine procedure. At the meantime, developmental work is being continued with the in situ hybridization and PCR (polymerase chain reaction) techniques, to refine these methods applicable in routine laboratory diagnosis. The complete HLA-typing of 100 follow-up women started earlier was concluded. In the statistical treatment of the vast amount of data accumulated during these 10 years, a progress was

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made with the development of the Prognostic Index, which could be used to predict the disease outcome more accurately than possible at the moment. Correlating the data obtained with the biological behaviour of the HPV lesions in these prospectively followed-up women, evidence on the factors regulating the clinical course of this increasingly common STD will be hopefully obtained. Tentative results are also available on immunohistochemical tests made to analyse the expression of a variety of HPV gene products (e.g., E6-encoded proteins) in the biopsies, using mono- and polyclonal antibodies generated against both synthetic peptides and fusion proteins. One of the future aims is directed towards the utilization of these data and such reagents in the development of serological tests for HPV infections. As a major part of the statistical analysis, the data derived from a case-control design (i.e., follow-up women and age-matched controls) were analysed to assess the factors increasing the risk for genital HPV infections. According to this analysis, the number of sexual partners during the past two years proved to be the single most important risk factor (RR >44 for women with 5 or more partners as compared to women with 1 partner only). Apart from the attempts to establish the prognostic factors for genital HPV infections, it will be of equal importance to outline the factors subjecting the people to clinical HPV infections which should be regarded as potentially premalignant conditions with significantly increased risk for genital cancer. Pertinent to this issue is another line of reserch initiated only recently; the exploration of the possible non-sexual transmission of HPV from an infected mother to her newborn baby. For that purpose, the babies and small children of the women in the follow-up are being examined for their HPV status in oral cavity and nasopharynx, by taking a swab to be analysed by PCR.

2.STUDIES CONDUCTED DURING THE BUDGET PERIOD (JANUARY-DECEMBER 1992)

Prospective Follow-up

During the budget period, the prospective follow-up of the women previously included in the series has been continued according to the original plans. The recruitment of new patients into the two Follow-up Groups A and B, has been terminated by the end of 1985; the final size of the cohort now being 530 women with HPV-infection (with or without concomitant CIN; HPV-CIN, and HPV-NCIN lesions, respectively). The mean follow-up time of the whole series approaches 80 months by now. During the ongoing period, a decision was made together with the clinical collaborators, that the

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follow-up of Groups A and B will be terminated during 1993-1994. This will be done as a highly organized manner by completing an end-point examination with extensive sampling and councelling for the future control of each individual woman. By that procedure and schedule, a mean follow-up time for the whole series becomes close to 10 years, which will be enough to elucidate the natural history of genital HPV infections.

Treatment

Starting from 1985, the third series of women has been recruited for this prospective follow-up study. This series is designated as the Treatment Group, currentiy comprising 530 women with HPV-CIN lesions. These patients are randomized into four distinct groups, each being treated with different modalities; conization, CO_2 laser vaporization, cryotherapy, and interferon treatment (topical administration as a cream). The close post-treatment follow-up of these women will disclose the efficacy of the above therapeutic measures on the clinical course of HPV infections. Most importantly, however, the study design permits the direct comparison between the natural history of this disease (i.e., spontaneous regression rate) and the clinical cure achieved with the active treatment. The follow-up of the Treatment Group women will be continued at least until the mean follow-up time will reach 5 years, i.e. until 1995-1996.

Examination of Samples

The women in all three series (A & B, Treatment Group) still attend the clinic at 6-month intervals, and on each attendance they are subjected to colposcopy, accompanied either by a directed punch biopsy and/or PAP smears. These are examined, as previously indicated, for the presence of HPV-induced cytopathic changes, concomitant CIN, HPV DNA by in situ hybridization and gene amplification by PCR. The role of accompanying cervical infections (possibly showing synergistic actions with HPV) is assessed by cervical swabs (for isolation of Chlamydia and HSV), and by serum samples for viral (HSV, CMV) serology. More recently, specific DNA probes and primers have become available which enable the direct detection of these agents in the biopsies or cellular swabs by ISH and PCR, respectively. In fact, our group has been involved in developing some of these tests into diagnostic use (see below).

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The fresh biopsy samples have been collected from all patients (at each attendance) and stored at -70°C for future analysis. So far, these have been used for Southern blot hybridization and for immunohistochemical staining of the immunocompetent cells (on frozen sections) within the local inflammatory infiltrate. Using monoclonal antibodies (McAb), the following cell types have been defined; B cells, T cell subsets, NK (natural killer) cells and Langerhans cells. These cells are currently being analysed also in the peripheral blood, using monoclonal antibodies and and flow cytometry by FACS. These data might aid elucidating some of the evidently complex immunological reactions involved in biology of HPV infections, recently accentuated by the attempts towards development of vaccines against papillomaviruses.

Oral Cavity as HPV Reservoir

During the ongoing research period, Dr. Jari Kellokoski, DDS, completed his PhD. Thesis which is the the first systematic approach to assess the biological significance of HPV infections in the oral cavity. The specific aims and results of the study were as follows: 1) To classify the morphological changes in the biopsies from buccal mucosa of women with genital HPV infection, and to evaluate the usefulness of oral scrapings, as well as morphological and histological criteria in assessing HPV infections in oral mucosa. **Results: By clinical inspection, the HPV association of tiny lesions is difficult to predict. On light microscopy, vacuolized cells were frequently encountered in clinically normal buccal mucosa. No correlation between vacuolized cells and kollocytes to HPV DNA content could be established.** 2) To analyse whether acetic acid staining can be applied on the specific diagnosis of oral HPV infections, and to further elucidate the factors ralated to the acetowhite staining, icluding the **smoking and drinking habits** and cytokeratin patterns in the epithelium. **Results: Acetic acid staining proved to be of no value in detection of HPV infection on oral mucosa. Instead, it was associated with smoking and seemed to be inversely age-dependent. In the biopsies containing HPV DNA, cytokeratin no.19 staining was intensified in the basal layer.** 3) To determine the sensitivity of differnet hybridization methods in detection of HPV infection and to oral HPV infections in buccal mucosa in women with genital HPV infections. Results: **Dot blot hybridization detected HPV DNA in 3.8% of the cases, while 21.8% proved to be positive with PCR. Dot blot positivity reflects only clinical and subclinical HPV infections. With Southern blot hybridization,**

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15.1% of biopsies taken from clinically normal buccal mucosa contained HPV DNA. 4) To assess HPV transmission by correlating the HPV types in the oral mucosa of the patients and those on the anogenital mucosa of their partners. **Results: Only a single patient had the same HPV type both in the genital and oral mucosa. Preliminary data on the anogenital HPV types in the male partners showed that none of them had the same HPV type as their female partners in the mouth, which suggested that transmission of HPV by oral-genital sex was unlikely.**

Non-Sexual Transmission of HPV

The above studies raised several questions which are the subject of the present study as follows: **1)** To explore the non-sexual routes of HPV transmission by examining the oropharyngeal swabs of newborn babies born to mothers with clinical HPV infections of the uterine cervix. **2)** To assess the clinical significance of such an eventual transmission of HPV (whether a true infection or a passenger) by sequential swabs of infants born to mothers with clinical HPV infections of the cervix. **3)** To analyse the clinical course of oral HPV infection in the infants. **4)** To establish the prevalence of HPV infections (e.g. skin warts, oral HPV infections, whether latent, subclinical or clinical) in children born to mothers with known genital HPV history. **5)** To correlate the HPV types in children to those of their parents.

The subjects to be studied are volunteer children born to women in the follow-up series since 1981. All children (appr. 250) are invited to attend the clinical examination for their oral HPV infections. The examination includes a clinical inspection, collection of saliva as well as oral scrapings to be analysed by PCR to detect HPV sequences. All clinical lesions will be biopsied. If the child is HPV-positive, then the virus typing data will be correlated to the HPV status of the parents. HPV-positive children will be followed-up for several years with regard to their oral mucosal lesions. The second study group consists of newborn babies (approx. 30) born to mothers with clinical HPV infection of the cervix. As control groups, a series of babies (about 50) born to healthy (i.e., non-HPV-infected) mothers, and 30 babies delivered by cesarean section will be included in the study. From all babies, a nasopharyngeal swap collected by suction immediately after delivery will be analysed by PCR to detect HPV DNA. All babies will be repeatedly analysed on the 4th day. The HPV DNA-positive babies will also be examined at the age of 6 weeks, 6 months and one year. The infants will be followed by oral scrapings. Pap

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smears will be taken from the mothers just before the delivery. This is the only study design, by which the eventual vertical transmission of HPV can be assessed, and the question whether HPV is a passenger or a true infective agent can be reliably answered.

Male Sexual Partners

The male sexual partners (Partner Group) of the women included in the Treatment Group are being recruited for examination, treatment and follow-up. Since the start of this recruitment in late 1986, the genital HPV lesions of >300 males have been examined by peniscopy (after acetic acid application), cytology, and biopsy including HPV DNA typing. Also the male HPV lesions are treated by laser, whenever appropriate. According to the data elaborated by Dr. Hippeläinen, the role of peniscopy as an important diagnostic tool in penile HPV lesions has been emphasized. It is important to realise, however, that peniscopy is not the conclusive diagnostic tool, because of its limited resolution, rendering it incapable of distinguishing between HPV and non-HPV lesions.

From the epidemiological standpoint, the concordance of HPV types between the sexual couples has become of prime importance. This concordance of a) genital HPV lesions and b) viral types in HPV-infected women and their male sexual partners, was analysed by Dr. Hippeläinen in her series of 282 women and their male partners examined by peniscopy and punch biopsy. Of the males, 199 (70.6%) showed histological evidence for HPV infection, 89.4% of which being flat lesions. HPV DNA was found in 181 (38.0%) of the 476 biopsies examined by ISH, exophytic warts (26 cases) and PIN lesions (20 cases) being most frequently HPV DNA-positive (92.3% and 80%, respectively). HPV DNA was also detected in 76 (31.3%) flat lesions histologically not equivocal HPV, i.e., in subclinical infections. Most importantly, of the 271 sexual couples subjected to HPV typing, both partners were ISH positive in 66 (24.4%) cases, but **only 15 (5.5%) couples had an identical HPV type** in their genital tract. The implications of these findings remain to be elucidated in the future studies.

One of the major aims of the partner study is to assess the effects of treatment given to the males on the clinical course of HPV-infections in their female sexual partners (included in the prospective follow-up series). The elucidation of this issue will also have major relevance in the proper evaluation

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op the treatment policy, and eventually might result in tremendous save of expenses, if the current hypothesis on the minor benefit achieved by the male treatment proves to be correct.

Asymptomatic Males

As a part of the male studies, a novel approach was made during the present study period, to determine (i) the prevalence of and (ii) risk factors for genital HPV infections in healthy males. Voluntary Finnish Army conscripts were examined using peniscopy after acetic acid, surgical biopsy, cytology and PCR for detection of HPV DNA in brush cytology samples collected from the urethra and the penile epithelium. The HPV data were complemented with a questionnaire inquiring their sexual habits. A total of 1471 (99.6%) males filled the questionnaire, and 432 of them attended the clinical study. The group entered in the examination differed from the nonattenders in that they reported more often genitourinary symptoms (p=0.000), had more sexual partners (p=0.002), and a previous history of sexually transmitted diseases (STDs) (p=0.000). Classical genital warts were present in 24 (5.6%) and papular lesions in 8 (1.9%) of the 432 men. Acetowhite epithelium was disclosed in 151/432 (35.0%) cases, of whom only 75/432 (17.4%) had obvious flat HPV lesions on peniscopy. Histological diagnosis of HPV infection was confirmed in 28 of the 42 (66.7%) biopsies taken from different types of lesions. HPV DNA was disclosed by PCR in 16.5% (47/285) of the adequate cell samples. Altogether, 26.2% (113/432) of the subjects were HPV-positive by at least one of the diagnostic methods, whereas only 6.5% (25/383) had a diagnostic peniscopic pattern and positive PCR. Furthermore, HPV DNA was found in 7.1% of the men with normal epithelium. In the logistic regression analysis, occasional sexual partners, previous STDs and no use of condom proved to be of predictive value for current HPV infection.

Prevalence and Incidence

The collection of the data on risk factors with suggested influence on transmission of the HPV infections has been continued by the detailed questionnaire for the sexual and smoking habits of the patients. Meantime, the annual mass-screening programme for cervical precancer lesions run by the Finnish Cancer Society Laboratory of Pathology in Kuopio has been utilized to define the prevalence of clinically manifest (i.e. detectable by PAP smear) genital HPV infections in unselected population of

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25-60 year-old women. The prevalence figures of 1991 were elaborated in early 1992, and the declining trend in disease prevalence noticed since the top year 1987 (1.77%) seems to continue, i.e. the figures falling down to 0.77% in 1991.

Risk Factors and Prognosis

The life-table method and log-rank analysis by Peto were used to assess the differences in clinical course between the groups of HPV lesions classified according to different variables; histologic grade, PAP smear grade, HPV type, colposcopic pattern etc. The survival tables were produced and the survical curves plotted with SPSS^{x} computer programme utilizing the product-limit (Kaplan-Meier) method. Estimation of reproducibility in diagnostic histopathology is also possible by applying the kappa statistics. This statistics takes into account the contribution of chance agreements. The intraobserver reproducibility and variation have been calculated for assessment of the punch biopsies and PAP smears.

The epidemiologist in our group, Dr. Vesa Kataja completed his doctoral Thesis in December. Im his work, the statistical techniques play a central role. The main purposes of his epidemiologic study were *a)* to establish the prevalence of cervical HPV infections in mass-screened Finnish female population aged 20-65 years, *b)* find out the incidence of the infection in a high-risk age group of 22-year old women, *c)* to elucidate the risk factors associated with clinical HPV infections, *d)* to assess the natural history of the disease during a long term prospective follow-up, and *e)* to establish prognostic factors predicting the disease outcome. The prevalence of cervical HPV infections was studied using cervical Pap smear in a conventional mass-screening in Kuopio County. The prevalence increased from 0.04% in 1981 to 1.76% in 1987, thereafter decreasing to 1.43% and 1.04% in two subsequent years. The prevalence varied significantly with age. The highest prevalence, 6.1%, was seen in the age group 20-29 years. In an extra mass-screening specially designed for a cohort of 22-year old women, the prevalence was studied in two successive years, 1985 and 1986. The prevalence in the first year was 2.7% and in the second year 7.1%, resulting in a crude annual incidence of 7.0% in this age group. Based on these figures, 79% of the Finnish female population would contract at least one genital HPV infection between the ages 20-79.

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The strongest independent risk factor for clinical HPV infection proved to be the increasing number of sexual partners within the past two years. Current smoking, previous CIN, STD and condyloma acuminatum were other factors associated with an increased risk for HPV infection. Regular use of IUD and good personal hygiene practices were among the factors with protective effect on the infection. During the mean follow-up of 79.2 months, 63.8% of the genital HPV infections (in 530 women) have spontaneously regressed without any treatment. Progression has occured in 13.8% of the cases. The strongest independent prognostic factors for progression were the high grade of CIN and HPV type 16. The adjusted relative risks for CIN I, CIN II and CIN III were 1.6, 2.9, and 9.6, respectively. The study provides basic epidemiologic data on genital HPV infections, which are the most prevalent STD of today. These data are also essential in defining the strategies for the management of these women.

Organotypic Culture as In Vitro Model

A significant impediment of HPV study has been the lack of a cell culture system that allows for expression of the complete viral life cycle. A cell culture system permissive for vegetative viral replication would be valuable not only to the understanding of PVs but also for the treatment and prevention of HPV-induced lesions. HPV life cycle has been shown to be coupled with the differentiation programme of keratinocytes, and a major obstacle to the propagation of HPV *in vitro* has been the inability to mimic the proliferation/differentiation cycles of HPV target tissue, the squamous epithelium. The organotypic culture system (raft) mimics the *in vivo* physiology of the epidermis by raising the cells to the air-liquid interface. This can be done either by the recombination of epidermal cells with dermal elements or through the use of a collagen matrix maintained on rigid support. Growing keratinocytes in either of these systems allows for a more complete differentiation programme to occur than is observed in monolayer.

To date, a number of modifications on the raft system exists. The basic technique used by most laboratories was introduced in 1984. In brief, fibroblasts are mixed with medium and collagen I at 0•C to 4•C and the placed into tissue culture dishes and incubated at 37•C until the solution solidifies. The collagen fibers and the fibroblasts form a lattice (plug) which is used as the dermal equivalent. Epidermal cells are seeded on top of this dermal equivalent, allowed to attach and form a monolayer.

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The plug is then lifted on to a metal grid, and fed so that the media comes only in contact with the bottom of the grid, maintaining the epidermal cells at the air-liquid interface. During the period of two to three weeks, the epidermal cells stratify and differentiate. The collagen plugs are then removed from the metal grids and prepared by standard techniques for histological examination.

The raft model has recently gained wide applications in HPV research. The utility of this system in our project was recognized recently, and facilities were offered to learn the technique in Prof. zur Hausen's laboratory in German Cancer Research Center, Heidelberg. A two-week visit there was done in early December by Dr. Stina Syrjänen, who is currently establishing the system in our laboratory.

Antisense Oligonucleotides in Experimental Therapy

Recent studies have focused on the ability of oligonucleotides to affect the genetic processes on many organisms, incuding viruses. Hence, oligonucleotides may represent a future source of biotechnologically derived compounds of therapeutic importance for several diseases such as cancer and viral infections. The viruses function through the transfer of genetic information from DNA to mRNA, and further to proteins. In case of retroviruses, the information originates from the viral RNA. These genetic processes can be intervented using oligonucleotides. The most widely used approach to interrupt the flow of genetic information is to use oligonucleotides as inhibitors of translation, with the complementary or "antisense" base sequence targeted to a specific "sense" sequence in the mRNA. Thus, expression of specific proteins can be regulated or inhibited. The preferred target for this antisense inhibition is the 5'-initiation codon. In general, an oligonucleotide of 15-20 bases is used for the antisense inhibition. The in vitro applications being successful so far include inhibition of expression of several oncogenes (c-myc, N-myc, myb) and HIV.

We have recently started a novel approach, where the ability of antisense oligonucleotides to inhibit the expression of HPV 16 and HPV 18 oncoproteins E6 and E7 18 is assessed in vitro. We have selected several antisense oligonucleotides to interact with target the mRNA of HPV 16 and HPV 18 in CaSki and HeLa cells, respectively. These oligos are automatically sulphurated (phosphorothiolate linkages) during the synthesis. The phosphorothiolate linkage makes the antisense oligos more

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resistent to nuclease degradation. We are using cationic lipids that form liposomes to facilitate the functional delivery of oligos into cells. Both the quantity of DNA and concentration of lipid required for optimal signals are being tested. According to our preliminary results, optimal conditions depend on the cell types used and differences exist in the reaction conditions between CaSki and HeLa cell lines. The interaction of liposomes with the DNA and the delivery of the antisense oligonucleotides into the cell are controlled by confocal laser scanning microscope (CLSM) by labelling the liposomes with rhodamin and the oligos with FITC. The effect of inhibition is assessed by analysing the expression levels of E6 and E7 mRNAs by Nothern blotting and quantitative RNA PCR. Extensive in vitro testing is a prerequicite for in vivo applications to HPV-infected patients.

Role of Anti-Oncogenes p53 and RB

The best known genes of this group are the retinoblastoma susceptibility gene (RB) and p53 gene. Both the RB protein and the p53 protein are interesting in that they are able of forming stable complexes with transforming proteins of several DNA tumor viruses. This would indicate the possibility that cellular tumor suppressor gene products may be inactivated by binding to viral oncoproteins. It has been shown that the E7 protein of HPV also has the ability of binding the RB protein and that the E7 protein shows sequences homologies to parts of the adenovirus EIA protein and the SV40 large T antigen involved in RB binding. Although the E7 protein of all HPVs can bind to RB, the E7 protein of HPV types associated with carcinomas, e.g. HPV 16 and 18, shows a higher affinity than the E7 gene product of HPV types found in benign lesions. It has also been demonstrated recently that HPV 16 E6 protein binds p53 tumor suppressor gene product, which usually acts as a negative regulator of cell proliferation.

The expression of tumor suppressor genes is changed in several tumors due to genetic alterations like deletions and mutations. In tumors the expression of mutant p53 is frequently increased when compared to wild-type p53 in normal cells. Due to the longer half-time of p53 expression as detected by immunohistochemistry is usually interpreted as the expression of mutant p53, not as the wild-type p53 expression present in cell cycle G_0-G_1 phase in proliferating cells. HPV 16 and 18 E6 protein binding to wild-type p53 has been found to enhance the degratation of the product by the ubiquitin-

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dependent protease pathway. The potency to form a complex with p53 diverges from a HPV-type to another. While the E6 proteins from HPV 16 and 18 bind to p53, the E6 proteins from HPV 6 and 11 serotypes fail to detectably bind with p53. This is of some interest because HPV 16 and 18 have been mostly found in cancers, therefore were called as 'high-risk' HPV types; while HPV 6 or 11 are most frequently associated benign condylomatous lesions.

Functional inactivation of wild-type p53, caused by either genomic mutations or neutralization by the transforming proteins from several tumor viruses, is currently regarded as an important molecular pathway for the pathogenesis of human malignancies. Theoretically, if HPV infections do play an etiological role in the pathogenesis of anogenital carcinomas, then one can easily estimate that cervical carcinomas associated with HPV should contain only a wild-type p53 gene (which would be neutralized and inactivated by binding to E6 protein), whereas cervical carcinomas without HPV would have mutant p53 genes (i.e. mutational inactivation of wild-type p53). This is indeed the case. Preliminary studies of cervical carcinomas and carcinoma derived cell lines have revealed point mutations within the p53 sequences only in HPV negative tumors. Thus, it seems that the inactivation of tumor suppressor genes may be a common mechanism for the transformation of cells shared by several DNA tumor viruses, including HPVs.

In our project, we have started to investigate the expression and mutations of p53 gene in dysplastic lesions and carcinomas immunohistochemically using PAb1801 antibody, which recognizes both the wild-type and mutant p53, as well as by p53 DNA sequencing with PCR amplification. Presence of HPV infection in these lesions will be demonstrated by PCR using HPV concensus (Manos et al 1990) and HPV 16 and HPV 18 type-specific primers. Amplified DNAs from PCR using concensus primers are blotted from agarose-gel onto filter and hybridized with biotinylated HPV-probe in low stringency to confirm the presence of HPV-DNA. Meanwhile, selected p53 DNA sequences in the lesions will be amplified by using specific primers chosen from the exon 5-9 (which are known as the most frequent area of point mutations) of the p53 genes. The amplified PCR products will be cloned into plasmid and subsequently sequenced using the chain-termination method, and the DNA sequences are determined by the automated DNA sequencer which is available at the Department of Biochemistry &

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Biotechnology. Expression level and gene sequence data of p53 will be compared with the presence of HPV-DNA. Similar techniques are being used to assess the role of RB gene expression and mutation. It will be of utmost interest to evaluate whether the detection or changes in these two anti-oncogenes are of any prognostic value in genital HPV lesions.

Development of Diagnostic DNA Tests

During the ongoing budget period, critical evaluations have been continued on the sensitivity and applicability in clinical use of the different DNA-hybridization techniques; Dot blot, Southern blot, in situ, as well as the role of PCR in diagnosis of HPV. These techniques have been extensively used in detecting and typing of HPV DNA in the clinical samples so far. To supplement the diagnostic pattern previously including the HPV DNA probes for HPV type 6, 11, 16 and 18, additional probes of HPV types have been acquired. At the moment, the **DNA probes of all known HPV types** are at our disposal. A systematic analysis (using ISH with seven different probes, HPV 6,11,16,18,31,33,42) of all the biopsies collected since 1981 was started in 1986. This technique is currently used in our laboratory as a routine method of typing the biopsies derived from the above four series of patients, and data are available of more than 4.000 biopsies by now.

As a part of our research programme, continuous developmental work has been done (as a joint effort with a Finnish biotechnology company, BIOHIT) for several years by now. A major goal of this joint venture has been to develop the laborous and complicated DNA hybridization techniques more applicable to routine pathology laboratory. During the year 1990, this line of research led into the first commercial products, when an in situ hybridization kit based on biotin-labelled whole genome HPV DNA probes was introduced to the international market (BioHit^{R}, HPV in Situ Typing Kit). This has subsequently led into the entire family of related products, including a sampling kit, a screening kit, as well as a selection of HPV-positive sample slides. This collaboration has been progressing also during this year, when the previously introduced kits for EBV (BioHit^{R} EBV in situ Test Kit), and the first non-radioactive dot blot test to screen and type HPV infections (BioHit^{H} HPV dot blot Screening Test and -Typing Test) were further improved. A new set of diagnostic tests is to be expected in the near future. These "second generation" HPV diagnostic reagents will be based on the use of synthetic

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oligonucleotide probes. The generation of such reagents in our laboratory became possible in June 1990, when a DNA synthesizer (Gene Assembler Plus, Pharmacia) was obtained in the premises. The technique to prepare the biotin-labelled oligonucleotide probes ready for ISH was recently developed in our laboratory (S.Syrjänen et al., J.Med.Virol. 1990).

Role of PCR

The gene amplification by PCR technique was introduced to our laboratory in early 1989, when the appropriate automatic PCR machine was acquired (Perkin Elmer Cetus, Thermal Cycler). PCR is currently recognized as the most sensitive technique of all, capable of disclosing less than a single copy of HPV DNA per cell. At the same time, however, there is a significant risk of false positive results due to DNA contamination (from a variety of sources). The successful application of the PCR technique also critically depends on the selection of correct primers. Because of the fact that most groups have used the primers designed by their own, considerable variation and discrepant results have been published of the detection rate of HPV by different laboratories. Accordingly, HPV DNA has been reported in from 2-3% to >80% of genital biopsies derived from 'normal epithelium'. According to our own experience, some 30% of the biopsies derived from the lower genital tract mucosa of the women with a normal recent PAP smear were shown to contain HPV DNA when subjected to PCR analysis (for HPV 6,11,16 and 18). Results in line with these have been also obtained while analysing the genital biopsies of males, where the HPV DNA detection rate by using PCR remains close to 30% even if their sexual partners have established HPV lesions. Beyond any doubt, then, much of the recent PCR data must be basically incorrect. Thus, it is clear that a substantial amount of standardization work in the technical aspects of the PCR method is necessary before the elucidation of its role in HPV diagnosis is possible. Such a work is continuously being done in our laboratory as well. As a recent progress in this field, an approach was developed in our laboratory, by which an accurate quantitation of the PCR amplification is possible.

Current Concepts and Future Outlook

A number of original reports, review articles and book chapters based on the results of the ongoing project have been published during the budget period (See the List of Reports). The results have also

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been presented in a number of international scientific congresses, as well as by giving invited guest lectures in a number of meetings abroad. Along with the extension of the prospective follow-up, our preliminary observations have been further confirmed suggesting that a certain proportion (about 15%) of the clinically manifest genital HPV infections, if left untreated, are capable of making definite progression, eventually into carcinoma in situ (CIS). These lesions thus present with the natural history identical with that of CIN lesions as established by well controlled prospective follow-up studies long before the association with HPV was recognized.

On the basis of the current understanding of the epidemiology of HPV infections, it would be of paramount clinical significance to establish the means to accurately predict the clinical course of HPV infections. Such a prediction should include the recognition of high risk lesions (i.e., those likely to make a rapid progression) from the low risk lesions liable to undergo a spontaneous regression without any treatment. To accomplish this, an extensive prospective study is mandatory. Our programme has provided some important data on this issue as well. During the 11-year follow-up (1981-1992), it has become apparent that the natural history of genital HPV-infections can be predicted (to certain extent) by the grade of the primary lesion at diagnosis. Accordingly, HPV lesions without CIN are more likely to regress sponatenously than those with CIN (HPV-CIN lesions). The latter, in turn, possess a high risk for progression as analysed by the life-table method. Along with the extension of the follow-up period, the dramatic difference originally suggested in the clinical behavior between the lesions infected by the low risk (HPV 6 and 11) and high risk (HPV 16,18,31,33 etc.) types has turned out to be less dramatic, albeit the highest risk for clinical progression seems to be associated with HPV 16 infections. In fact, the risk of progression for HPV 16 lesions seems to be more than 5-fold as compared with that of HPV 6- or HPV DNA-negative lesions. HPV type, on the other hand, could not be used to accurately predict the lesions with eventual spontaneous regression, albeit the regression rate of HPV 16 lesions seems to be very low indeed. At present, too little data are availabe on the biological behavior of the infections by the more recently described HPV Types 31, 33, 35, 39 and 42, and of the 50- and 60-series, some of which have been shown to be associated with the high grade CIN lesions and cervical cancer. **To fully elucidate the prognostication of HPV infections, more data are needed about the factors involved in regulation of the viral life cycle within the host cells,**

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including the growth factors, their receptors, as well as the complex interplay between the divergent hormonal factors, oncogenes, anti-ongcogenes etc. Once again, the full understanding of these mechanisms **necessitates a long-term prospective study** with extensive patient material. Such a set-up is available in Kuopio, where hopefully this follow-up project can be continued for a number of years ahead.

3.RESEARCH INVOLVING HUMAN SUBJECTS

The study protocols performed in the past year for human subjects in Group A and B have been explained in detail in the grant application. Because these protocols have remained the same, they will be explained only shortly in this context. As detailed for the subjects in Group A and B, the HPV-positive PAP smear is the single and only criteria of their inclusion in the study. The same is true with the recruitment of the women into the Treatment Group. Only the women presented with unequivocally HPV-positive smears containing changes consistent with CIN are invited in the Treatment Group. This practice is in full agreement with the general policy in Finland, according to which all women with cytological changes suggesting CIN should be referred to a colposcopic examination, independent of whether HPV infection is present or not.

The recruitment of the subjects into the three study groups was accomplished by an invitation letter, mailed along with the pathologist's report of the PAP smear. Thus, it is completely up to the patient to decide if she wants to enter the study and also to terminate the follow-up at any stage of the study. The detailed characterization of the specimens as well as the means of obtaining them have been given in the grant application, and these procedures in the Treatment Group do not differ in any way from those of Group A and B. Thus, the collection of the research and diagnostic material in the present study is accomplished by utilizing well established techniques in worldwide diagnostic use to monitor the genital infections and precancerous lesions.

The treatment modalities instituted to the women in the Treatment Group only include the established treatment techniques; conization, CO_2-laser vaporization therapy, cryotherapy, as well as interferon (as a topical cream or s.c.injections), the efficacy of which is currently being explored in many laboratories.

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As in any diagnostic and therapeutic clinical work, certain minor risks exist, which are unpredictable. During the 10-year existence of this project, however, no major complications have developed in any of the patients. It is completely clear, then, that the patients in the present three series are not subjected to any risk major than due to the diagnostic procedures used (colposcopy, PAP smear, punch biopsy, cervical swab, serum sampling, interview anonymously by a questionnaire), as well as due to the therapeutic procedures (conization, laser, cryo, and interferon). No alternative procedures are available to conduct an adequate prospective follow-up of genital HPV lesions (either non-treated or treated) other than those used in the present study, and repeated at intervals regarded safe to ensure that none of the HPV lesions will escape an early detection and proper treatment, whenever making progress.

The above diagnostic and therapeutic protocols also apply to the male sexual partners included in the Partner Group. This is also true with their continuous monitoring by the gynecologists responsible for their examination in the Outpatient Department of Gynecology and Obstetrics, Kuopio University Hospital. The above diagnostic protocols apply equally to the newborn babies and children enrolled for oral examinations since June 1992, after being accepted by the Ethical Committe of Kuopio University.

As previously emphasized, the risks to which the present patients are subjected are limited to those due to the application of the commonly accepted diagnostic and therapeutic procedures. The anticipated benefit of the patients while included in this most carefully controlled prospective follow-up study significantly exeeds the neglible risks to be expected from the use of colposcopy, PAP smears, punch biopsies, cervical swabs and serum sampling as well as conization, laser, cryo or interferon cream treatment. This benefit will be even accentuated, when the follow-up results are compared with the clinical outcome of the HPV lesions in non-controlled or even in mass-screened female population, subjected to PAP smears irregularly or at 5-year intervals, respectively. This is self-evident also for the males, now being examined and treated, but who, outside this study usually present with subclinical lesions indiscernible without colposcopy, and as such form a potential reservoir of HPV infections to be transmitted to their sexual partners.

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4.REPORTS PUBLISHED DURING THE BUDGET PERIOD (JANUARY-DECEMBER 1992)

A) Original Reports

1.Syrjänen, S., Sainio, P., Chang, F., Johansson, B. and Syrjänen, K. Epstein-Barr virus (EBV) genomes and c-myc oncogene in oral Burkitt's lymphomas. Scand. J. Dental. Res. 100, 176-180, 1992.

2.Yliskoski, M., Saarikoski, S. and Syrjänen, K. Conization for CIN associated with Human papillomavirus infection. Arch. Gynecol. Obstet. 249, 59-65, 1991.

3.Yliskoski, M., Tervahauta, A., Saarikoski, S., Mäntyjärvi, R. and Syrjänen, K. Clinical course of cervical Human papillomavirus lesions in relation to coexistent cervical infections. Sex. Transm. Dis. 19, 137-139, 1992.

4.Kataja, V., Syrjänen, K., Syrjänen, S., Mäntyjärvi, R., Yliskoski, M., Saarikoski, S. and Salonen, J.T. Prognostic factors in cervical human papillomavirus (HPV) infections. Sex. Transm. Dis. 19, 154-160, 1992.

5.Syrjänen, S., Andersson, B., Juntunen, L., Syrjänen, K.J. Polymerase chain reaction for producing biotinylated Human papillomavirus DNA probes for in situ hybridization. Sex. Transm. Dis. 19, 140-145, 1992.

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7.Syrjänen, K. Human papillomavirus infection of the female genital tract. Editorial. Cytopathol. 2, 225-227, 1991.

8.Syrjänen, K.J. Genital human papilloma virus infections in HIV-infected males and females. In: Melica F (ed). AIDS and Human Reproduction, Karger, Basel, pp. 74-83, 1992.

9.Kataja, V., Syrjänen, S., Yliskoski, M., Hippeläinen M., Väyrynen, M., Saarikoski, S., Mäntyjärvi, R., Jokela, V., Salonen, JT, and Syrjänen, K. Risk factors associated with cervical human papillomavirus (HPV) infections: A case-control study. Am. J. Epidemiol. Submitted and Revised.

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12.Tuppurainen, K., Raninen, A., Kosunen, O., Kankkunen, J-P., Kellokoski, J., Syrjänen, S., Mäntyjärvi, M. and Syrjänen, K. Squamous cell carcinoma of the conjunctiva. Failure to demonstrate HPV DNA by in situ hybridization and polymerase chain reaction. Acta Ophthalmol. 70, 248-254, 1992.

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14.Tervahauta, A., Syrjänen, SM., Kallio, PJ. and Syrjänen, KJ. Immunohistochemistry, in situ hybridization and polymerase chain reaction (PCR) in detecting c-myc expression in human malignancies. Anticancer. Res. 12, 1005-1011, 1992.

15.Tosi, P., Cintorino, M., Santopietro, R., Lio, R., Barbini, P., Ji, H., Chang, F., Kataja, V., Syrjänen, S. and Syrjänen, K. Prognostic factors in invasive cervical carcinomas associated with human papillomavirus (HPV): Quantitative data and cytokeratin expression. Pathol. Res. Pract. 188, 866-873, 1992.

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18.Syrjänen, K.J. The effect of viruses on tumor induction. In: Chemical Induction of Cancer (CIC). ed. J. Arcos. Submitted.

19.Syrjänen, S. and Syrjänen, K. Human papillomavirus (HPV) infections of the genital tract. Clinical Significance and diagnosis by PCR. In: Frontiers in Virology: Diagnosis of Human Viruses by Polymerase Chain Reaction Technology. (eds). Y. Becker, G. Darai, Springer-Verlag, Heidelberg. pp 185-207, 1992.

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21.Syrjänen, K.J. Papilloma virus and cervical cancer. Vox 14, 3-12, 1992.

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23.Costa, S., Syrjänen, S., Vendra, C., Chang, F., Guida, G., Tervahauta, A., Hippeläinen, M. and Syrjänen, K. Detection of Human papillomavirus infections in the male sexual partners of women attending an STD clinic in Bologna. Int. J. STD & AIDS. 3, 338-346, 1992.

24.Syrjänen, K.J. Does Human papillomavirus (HPV) cause cervical cancer? Int. J. Clin. Virol. 1, 2-6, 1992.

25.Chang, F., Wang, L., Syrjänen, S. and Syrjänen, K. Human papillomavirus (HPV) infections in the respiratory tract. Am. J. Otolaryngol. 13, 210-225, 1992.

26.Syrjänen, K.J. The normal cervix. Concept of the transformation zone. Proceeding of the 1st Europaen Course on genital HPV-associated pathology. Cervix & I.f.g.t. 10, 83-88, 1992.

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30.Syrjänen, K.J. HPV typing: Does it help or not? Proceedings: Nordisk Förening för Ostetrik och Gynekologi, XXVIII Kongress. Reykjavik, Iceland, June 9-12, 1992. Submitted.

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35.Chang, F., Syrjänen, S., Kurvinen, K. and Syrjänen, K. The p53 tumor-suppressor gene as a common cellular target in human carcinogenesis. Am. J. Gastroenterol. In press.

36. Chang, F., Syrjänen, S.M., Wang, L. and Syrjänen, K. Infectious agents in the etiology of esophageal cancer. Gastroenterology 103, 000-000, 1992.

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B) Abstracts

1.Syrjänen, K. Viral infections and genital cancer. The VIIth Meeting of the Scandinavian Society for Genitourinary Medicine (SSGU), Trondheim, Normay, February 13-16, 1992.

2.Syrjänen, S., Kellokoski, J., Yliskoski, M., Syrjänen, K. Oral HPV infections in women with genital HPV lesions. Evaluation of the clinical, morphological and HPV DNA findings. The VIIth Meeting of the Scandinavian Society for Genitourinary Medicine (SSGU), Trondheim, Normay, February 13-16, 1992.

3.Syrjänen, K. Papilloomavirukset: DNA-Diagnostiikan luotettavuus ja tarpeellisuus. XIX Tuusula Symposium. Gynekologiset Syövät. Tuusula, 6-7.2.1992.

4.Yliskoski, M., Hippeläinen, M., Kataja, V., Saarikoski, S., Syrjänen, K. Pregnancy and natural history of cervical HPV. 7th European Congress of Gynecology and Obstetrics, Helsinki, June 28.- July 1, 1992.

5.Yliskoski, M., Saarikoski, S. and Syrjänen, K. Conization for CIN associated with Human papillomavirus infection. Ob. Gyn. Digest. In press.

6.Chang, F., Syrjänen, S., Shen, Q., Wang, L., Wang, D. and Syrjänen, K. Human papillomavirus (HPV) involvement in esophageal precancerous lesions and squamous cell carcinoma as evidenced by microscopy and different DNA-techniques. Symposium on Human Tumorviruses, Heidelberg, Germany, May 3-6, 1992.

7.Chang, F., Syrjänen, S., Shen, Q., Wang, L., Wang, D. and Syrjänen, K. Human papillomavirus (HPV) involvement in esophageal precancerous lesions and squamous cell carcinoma as evidenced by microscopy and different DNA-techniques. International Congress on Cancer of the Esophagus, Santa

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8.Chang, F., Syrjänen, S., Wang, L. and Syrjänen, K. Preliminary results of the mass screening of human papillomavirus (HPV) infections in esophageal squamous cell carcinomas by in situ hybridization with biotinylated HPV DA probes in archieval specximens from the high-incidence area of China. 11th International Papillomavirus Workshop, Edinburgh, Scotland, September 5-11, 1992.

9.Chang, F., Syrjänen, S., Tervahauta, A., Kallio, P., Wang, L. and Syrjänen, K. Detection and characterization of DNA sequence homogeneous to human papillomavirus (HPV) genomes from esophageal squamous cell carcinomas. 11th International Papillomavirus Workshop, Edinburgh, Scotland, September 5-11, 1992.

10.Syrjänen, K. Epidemiology of HPV infections and genital neoplasia. First International Congress for Infectious Diseases in Obstetrics and Gynecology, Taormina-Giardini Naxos, June 14-19, 1992.

11.Syrjänen, K. Genital HPV infections in etiology of cervical cancer. XXth European Congress of Cytology, Prague, Czechoslovakia, September 23-26, 1992.

12.Syrjänen, S. and Syrjänen K. Papilloomavirusinfektio: Syövän aiheuttaja vai harmiton tulehdustauti? Dialogia Tiedotuslehti 3: 11-15, 1992.

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14.Syrjänen, K. Virukset ja syöpä. Kirjassa: Suomen Syöpäinstituutin Säätiö, s. 16-17, 1992.

15.Ji, HX., Yliskoski, M., Väyrynen, M., Hippeläinen, M., Syrjänen, S., Syrjänen, K. Analisi colposcopica delle infezioni da papillomavirus genitale umano condotta con controlli prospettici durati otto anno. Giorn. It. Ost. Gin. 3, 216-224, 1992.

16.Syrjänen, K. HPV and CIN: Histopathological assessment. 7th Congress of the Italian Society of Colposcopy and Cervigo-Vaginal Pathology, Bologna, December 14-15, 1992.

17.Syrjänen, K. Risk factors in HPV-associated cervical carcinogenesis. 7th Congress of the Italian Society of Colposcopy and Cervigo-Vaginal Pathology, Bologna, December 14-15, 1992.

C) Doctoral (Ph.D.) Thesis

1.**Kellokoski J.** Human Papillomavirus (HPV) Infections of the Oral Cavity in Women with Genital HPV Infections. Publications of the University of Kuopio. Dental Sciences, Original Reports 1/1992.

2.**Kataja V.** Genital Human Papillomavirus (HPV) Infections. Prevalence, Incidence, Risk Factors and Prognosis. Kuopio University Publications. Medical Sciences, Original Reports 13/1992.

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4.PROJECT BUDGET, January - December 1992

Items	Costs (FIM)
Technical Assistants (Full time, 2 x 12 months): Mrs. Helena Kemiläinen (1.1.-31.12.92) Mrs. Kaarina Hoffren (1.1.-31.12.92)	275.000,-
Computer Operator, (Full time, 1 x 12 months): Ms. Paula Lipponen (1.1.-31.12.92)	125.000.-
Material costs: Laboratory chemicals, reagents, isotopes, immunohistochemical kits, DNA reagents etc.	318.350.-
Printing costs: Reprints, page charges, colour prints	22.500.-
Congress costs: Travel expenses, congress fees, accomodation	10.500,-
Total Costs	751.350.- (FIM)
(USD 80.000,- + USD 95.000,-)	175.000.- (USD)

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